NON-LEAF CHLORENCHYMA IN *BIENERTIA CYCLOPTERA* AND *SUAEDA ARALOCASPICA* (CHENOPODIACEAE) EXHIBIT SINGLE CELL C_4 PHOTOSYNTHESIS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of CHRISTINE NICOLE CLAY find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Both *Bienertia cycloptera* and *Suaeda aralocaspica* (Chenopodiaceae) are terrestrial C_4 plants with leaves that lack traditional C_4 anatomy. Instead, each has a unique single cell C_4 system which utilizes individual chlorenchyma cells by means of intracellular biochemical and organelle compartmentation. Anatomical and immunocytochemical analysis of *B. cycloptera* flowers and stems shows that the chlorenchyma cells have the same organization as those in leaves, with the chloroplasts of the central cytoplasmic compartment containing Rubisco and PEPC localized to the cytoplasm. The flowers of *S. aralocaspica* also have the same chlorenchyma organization as the leaves, with chloroplasts polarized towards opposite ends of the cells. Rubisco is found toward the proximal end of the cell (towards the vascular tissue) and PEPC is found throughout the cytoplasm. In the stems of *S. aralocaspica* there is no distinct layer of chlorenchyma cells, nor are there chlorenchyma cells with the distinct polarization of chloroplasts. Instead, there are some chlorenchyma cells scattered throughout the cortical tissue which have chloroplasts around the periphery of the cell, typical of C_3 type chlorenchyma. Western blot analysis of the flowers showed that the C_4 enzymes Rubisco, PEPC, PPDK, and

NAD-ME are present in similar levels to those found in the leaves of both species. Rates of photosynthesis in flowers measured by $^{14}CO_2$ uptake on a chlorophyll basis were similar to previously measured rates on leaves for both species.

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Dedication

This thesis is dedicated to the memory of Dr. Vince R. Franceschi

who recruited and inspired me.

CHAPTER ONE

INTRODUCTION

Photosynthesis needs no advocate to defend its status of being fundamentally essential to plants. Its importance to humans is evidenced by the long list of necessities including oxygen, fossil fuels, chemicals, and medicines derived from plant processes supported by or directly related to photosynthesis (Hall, 2001). Photosynthesis is a complex process that, though first investigated in the late 1700s, continues to present novel challenges to researchers. For plants to perform successful photosynthesis there have been adaptations to environmental conditions (e.g. temperature, light, salinity, and drought). Differences in mechanisms and regulation are often used to identify the three forms of photosynthesis known in higher plants— C_3, C_4, and Crassulacean Acid Metabolism (CAM).

Most photosynthetic organisms including photosynthetic bacteria, cyanobacteria, algae, and terrestrial plants have a common mechanism of carbon assimilation through the carboxylase enzyme ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) and the C_3 pathway which consists of 13 enzymatic steps. This enzyme functions both as a carboxylase and oxygenase (equations below).

$$\text{RuBP} + CO_2 \xrightarrow{v_c} 2\ \text{3-phosphoglycerate}$$

$$\text{RuBP} + O_2 \xrightarrow{v_o} \text{3-phosophoglycerate} + \text{P-glycolate}$$

where v_c = velocity of carboxylase and v_o = velocity of oxygenase.

According to current scientific hypotheses, when photosynthesis first came into being the CO_2 concentration in the atmosphere was estimated to be at least an order of magnitude higher than what it is today, while the O_2 concentration was considered to be negligible. With such high

levels of CO_2 and so little O_2, the substrate CO_2 was likely saturating for carboxylase activity, and little or no oxygenase activity occurred. As atmospheric levels of CO_2 declined and O_2 increased, conditions became more favorable for oxygenase activity. Reaction of CO_2 relative to O_2 is defined by the equation below.

$$\frac{v_c}{v_o} = S_{rel} \frac{[CO_2]}{[O_2]}$$

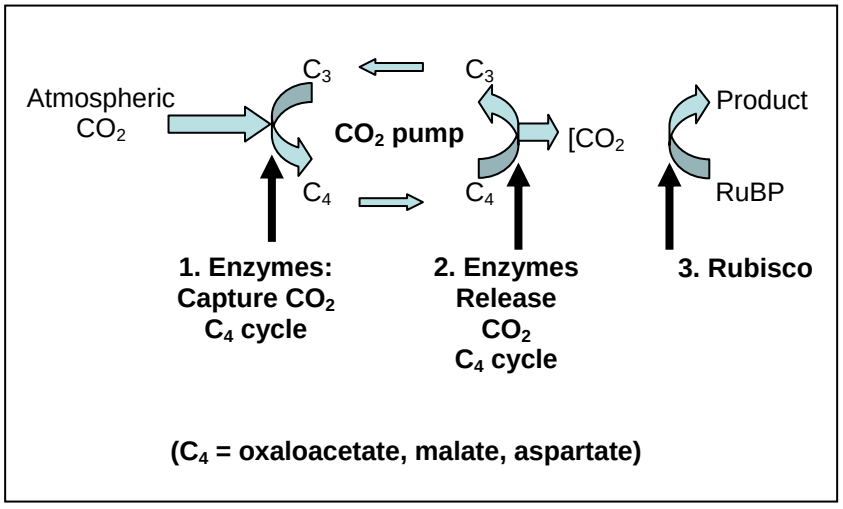
where v_c = velocity of carboxylase, v_o = velocity of oxygenase, and S_{rel} = specificity factor for reaction with CO_2 relative to O_2. The consequence of reaction with O_2 is the production of phosphoglycolate which is converted to glycolate and P_i through P-glycolate phosphatase. Organisms either excreted glycolate or employed a pathway to recover some of the carbon from the glycolate pathway. In the glycolate pathway carbon is metabolized through peroxisomes and mitochondria resulting in synthesis of glycerate which can reenter the C_3 cycle in the chloroplasts by its phosphorylation to form 3-PGA (through PGA kinase). While this leads to a recovery of 75% of the carbon, the glycolate pathway results in photorespiration; CO_2 is lost through glycine decarboxylase in the mitochondria, and O_2 is consumed. Energy is utilized through reduction of the 3-PGA formed in the chloroplasts and reassimilation of ammonia generated through glycine decarboxylase. A simple equation describing the net rate of CO_2 fixation with respect to Rubisco is shown below where A = net rate of CO_2 uptake, v_c = velocity of carboxylase, and v_o = velocity of oxygenase.

$$A = v_c - 0.5\ v_o$$

Obviously, due to the competitive interaction of CO_2 and O_2, a decrease in CO_2 levels and an increase in O_2 levels in the atmosphere would result in increased photorespiration and decreased net rates of CO_2 uptake. Also, increasing temperatures become more favorable for oxygenase activity due to a decrease in the affinity of Rubisco for CO_2 (increase in K_m). Thus aquatic

organisms like cyanobacteria and microalgae are benefited by CO_2 concentrating mechanisms consisting of membrane-associated inorganic carbon pumps and carbonic anhydrases (which convert bicarbonate to CO_2 for utilization by Rubisco). At the beginning of terrestrial plant life CO_2 levels in the atmosphere were considered sufficient to limit photorespiration. Therefore, atmospheric CO_2 diffuses into the leaf to the chlorenchyma cells where it is directly fixed by Rubisco in the C_3 cycle with the first product of photosynthesis 3-phosphoglycerate. Subsequently, these plants were named C_3 plants.

The continual decline in atmospheric levels of CO_2 combined with higher temperatures in some biomes made a CO_2 concentrating mechanism favorable. This CO_2 concentrating mechanism is a biochemical CO_2 pump called the C_4 pathway which is illustrated below.



The key to the efficiency of C_4 photosynthesis lies in the spatial separation of reactions associated with C_4 acid synthesis and C_4 acid decarboxylation with donation of CO_2 to Rubisco (step 1 versus steps 2 and 3). Traditional C_4 plants separate steps between two cell types with C_4 acid synthesis in the mesophyll cells which surround the bundle sheath cells where the C_4

decarboxylation and Calvin cycle occur. This is known as Kranz anatomy (Edwards and Walker, 1983).

Thus far C_4 plants have been identified in 19 angiosperm plant families (Table below).

Angiosperm families	
Acanthaceae	*Gisekiaceae*
Aizoaceae	*Hydrocharitaceae*
Amaranthaceae	*Molluginaceae*
Asteraceae	*Nyctaginaceae*
Boraginaceae	*Poaceae*
Brassicaceae	*Polygonaceae*
Caryophyllaceae	*Portulacaceae*
Chenopodiaceae	*Scrophulariaceae*
Cyperaceae	*Zygophyllaceae*
Euphorbiaceae	

(Sage, 2004)

Among the 19 families, there are different types of Kranz anatomy and C_4 biochemistry. There are three types of C_4 cycles based on differences in the C_4 acid decarboxylase which donates CO_2 to Rubisco. These are (1) NADP-malic enzyme which occurs in the bundle sheath chloroplasts, (2) NAD-malic enzyme which occurs in bundle sheath mitochondria, and (3) PEP-carboxykinase which occurs in the cytosol in bundle sheath cells (Edwards and Walker, 1983; Sage, 1999). About half of the species in family Poaceae are C_4, many species in family Cyperaceae are C_4, and among dicots the family Chenopodiaceae has currently been found to have the most C_4 species. The Chenopodiaceae family has five types of Kranz anatomy including Atriplicoid, Conospermoid, Kochioid, Salsoloid, and Suaedoid, and the two biochemical types NADP-ME and NAD-ME (Voznesenskaya and Gamaley, 1986).

Another C_4 pathway of photosynthesis, called Crassulacean Acid Metabolism (CAM), occurs in many plant families where the potential for water loss during the day is very high (See Table below).

Major families	Minor families
Agavaceae	Apocynaceae
Aizoaceae	Asteraceae
Asclepiadeaceae	Commelinaceae
Asphodelaceae	Cucurbitaceae
Bromeliaceae	Dracaenaceae
Cactaceae	Geraniaceae
Clusiaceae	Gesneriaceae
Crassulaceae	Lamiaceae
Didiereaceae	Oxalidaceae
Euphorbiaceae	Passifloraceae
Orchidaceae	Piperaceae
	Polypodiaceae
	Portulaceae
	Rubiaceae
	Vitaceae
	Vittariaceae
	Welwitschiaceae

(Winter and Smith, 1996)

In order to reduce transpiration, CAM plants open their stomates at night and convert CO_2 to malate via PEP carboxylase and NAD- or NADP-malic dehydrogenase. During the day the stomates are closed and malic enzymes or PEP carboxykinases convert malate to CO_2 for the Calvin cycle. Therefore, the fixation of CO_2 by the C_4 cycle and donation of CO_2 from C_4 acids to Rubisco are separated temporally.

Terrestrial plants which live in temperate or cooler climates with sufficient water have sufficient CO_2 available which minimizes the level of photorespiration. However in other conditions, e.g. high temperature, drought and salinity, where CO_2 can be limiting, the efficiency of photosynthesis in C_3 plants can drop dramatically. Yet C_3 is found in nearly all economically important crop species, including rice and wheat. C_3 crops which grow in warmer climates and/or with limiting water might be improved by conversion to C_4 photosynthesis.

The main reduction in efficiency of C_3 plants is due to limited supply of atmospheric CO_2 to Rubisco. This limitation leads to high photorespiration—a displacement of CO_2 by oxygen in the Calvin cycle leading to the release of CO_2 instead of oxygen (Decker, 1955).

The existence of the more efficient C_4 photosynthesis, though limited to 3% of all land plants (Sage and Monson, 1999), has stimulated a desire within the scientific community to improve the efficiency of photosynthesis in crop plants by genetic engineering for C_4 mechanisms (Sheehy et al., 2000). C_4 plants are considerably more efficient than C_3 plants under various conditions such as high temperature, water stress, low atmospheric CO_2, and high salt concentrations (Caldwell et al., 1977; Ku and Edwards, 1978; Ehleringer et al., 1991).

Until recently, C_4 photosynthesis was thought to be dependent on Kranz anatomy for enzymatic segregation (Voznesenskaya et al., 2001; Sage, 2002; Voznesenskaya et al., 2002; Edwards et al., 2004). Current findings have challenged this paradigm, with the discovery of several plants in the family *Chenopodiaceae* that undergo C_4 photosynthesis but have a unique anatomy that does not consists of both mesophyll and bundle sheath cells. Instead, the single cell C_4 system utilizes individual chlorenchyma cells by means of intracellular biochemical and organelle compartmentation (Voznesenskaya et al., 2001). It has been demonstrated that the mature leaves of *Bienertia cycloptera*, *Bienertia sinuspersici*, and *Suaeda aralocaspica* perform this exceptional form of photosynthesis by separate means of partitioning intracellular functions (Voznesenskaya et al., 2001; Freitag and Stichler, 2002; Voznesenskaya et al., 2002; Edwards et al., 2004; Akhani et al., 2005).

Both *B. cycloptera* and *S. aralocaspica* are native to Central Asian deserts and semi-deserts (Akhani et al., 1997; Freitag, 2000; Akhani, 2003). C_4 photosynthesis is more efficient than C_3 under the arid conditions because as the temperature increases the K_m of CO_2 increases,

and the solubility of gases in the aqueous phase decreases with that of CO_2 decreasing more rapidly than O_2. The increase in the solubilized O_2/CO_2 ratio causes photorespiration to occur in C_3 plants as O_2 and CO_2 compete for binding sites on Rubisco (Ku and Edwards, 1978). C_4 plants largely avoid photorespiration at higher temperatures due to their CO_2-concentrating mechanism (CCM).

Efficient photosynthesis in C_4 plants results from both the success of their CCM, and their ability to refix photorespiratory CO_2. As noted earlier, photorespiration occurs when O_2 binds to ribulose-1, 5-bisphospate (RuBP). Instead of being carboxylated by Rubisco, RuBP is oxygenated and the result is a release of CO_2 through metabolism in the glycolate pathway. Refixing any photorespired CO_2 is possible in C_4 plants due to their Kranz anatomy. By sequestering Rubisco in the bundle sheath cells and importing C_4 acids as generators of CO_2 from the surrounding mesophyll cells, C_4 plants concentrate CO_2 around Rubisco which represses photorespiration. There may be little depression of O_2 levels except in some C_4 species which have bundle sheath chloroplasts lacking PSII activity and water oxidation (NADP-ME type C_4 plants).

A further benefit of Kranz anatomy is that glycine decarboxylase (GDC) is specifically located in the mitochondria of bundle sheath cells so that any CO_2 generated from photorespiration can be refixed by Rubisco. O_2 which diffuses from the atmosphere to bundle sheath cells can result in some oxygenation of RuBP and production of glycine. GDC releases CO_2 from glycine which can then be refixed by Rubisco.

The plants which have single-cell C_4 photosynthesis, *B. cycloptera, B. sinuspersici,* and *S. aralocaspica,* are not confined by this traditional model of C_4 anatomy. In the single cell C_4 system of *B. cycloptera* and *B. sinuspersici*, the peripheral compartment of the chlorenchyma

assumes the role of the mesophyll cells in Kranz anatomy, while a unique central cytosolic compartment replaces the function of bundle sheath cells in Kranz anatomy. The peripheral compartment contains chloroplasts with the enzyme pyruvate, Pi dikinase (PPDK), which is necessary for generating PEP from pyruvate for CO_2 fixation by PEPC and conversion of photosynthetic intermediates to malate, while Rubisco, NAD-malic enzyme (NAD-ME), and GDC are restricted to the central cytosolic compartment where NAD-ME catalyzes the decarboxylation of malate in mitochondria to produce CO_2 for the C_3 cycle (Voznesenkaya et al., 2002).

In the single cell C_4 plant *S. aralocaspica* the chlorenchyma cells are very elongated with dimorphic chloroplasts polarized toward opposite ends of the cell. The function at the distal end where atmospheric CO_2 enters the leaf replaces that of mesophyll cells in the Kranz system (PPDK-containing chloroplasts converting pyruvate to PEP, the substrate for PEP carboxylase), whereas the proximal region replaces that of the bundle sheath where C_4 acids are decarboxylated by mitochondrial NAD-ME and CO_2 is donated to Rubisco-containing chloroplasts. Immunolocalization studies show Rubisco and NAD-ME in the proximal end of the cell, PPDK in the distal chloroplasts, and PEPC distributed in the cytoplasm and enriched in the distal end (Voznesenkaya et al., 2001).

The advantages and disadvantages of the single cell C_4 system in *B. cycloptera* and *S. aralocaspica* are only beginning to be studied. Is this just an alternative means to perform C_4 with the same efficiency as that in Kranz type C_4 plants? Does having the C_4 mechanism in a single photosynthetic cell allow it more plasticity to potentially switch between C_3 and C_4 than in the Kranz type C_4 which generally is an obligate C_4 system? For C_4 to function effectively, the CO_2 generated by decarboxylation of C_4 acids must have sufficient resistance to loss by diffusion

to favor its capture by Rubisco. In this respect, is the design of single cell C_4 systems as effective as the Kranz type? Intuitively, it seems that producing a single cell type could be energetically more favorable considering structural differences, but this has yet to be proven. Kranz anatomy requires producing two photosynthetic cells, with thick-walled bundle sheath cells. For researchers, the single cell system has the potential to allow the engineering of C_4 photosynthesis in C_3 plants without having to engineer Krantz anatomy.

The main focus of this research was to determine if all green cells in different organs in these species participate in single cell C_4 photosynthesis. While leaves are an important photosynthetic tissue, casual observation of *B. cycloptera* and *S. aralocaspica* reveals that the young stems are green, and flowers with green tepals are abundant during latter stages of growth. Pictures of these species during reproductive growth under natural conditions show that flowers are abundant and green (Freitag, personal communication to G. Edwards; www.alsirhan.com, 2002). If photosynthetic, they could make a significant contribution to carbon assimilation.

S. aralocaspica

B. cycloptera

Also, it is possible under severe stress in semi-arid conditions that leaves are lost leaving stems and flowers to provide the main source of photosynthate (Akhani, personal communication). It was previously shown that some of the C_4 chenopods in subfamily Salsoloideae have leaves which are C_4 and cotyledons which are C_3 (Pyankov et al., 2000b). Whether it is beneficial for different organs to have C_3 versus C_4 photosynthesis could depend on climate conditions. Germination in the spring under cooler, moist conditions could be favorable for C_3 photosynthesis in cotyledons. C_4 photosynthesis in flowers in the fall under hot, dry conditions could be favorable, whereas C_3 could be favorable under cooler, moist conditions. Other factors may include the energetics of producing C_3 versus C_4 chlorenchyma tissue (C_4 may cost more: two types of chloroplasts, two cytoplasmic compartments, cytoskeleton development, C_4 and C_3 enzymes), or there may be genetic constraints such that all photosynthetic tissue produces the same type of photosynthetic structure/mechanism. The anatomy of the green non-leaf tissues may give clues as to the process by which the single cells differentiate. Although the leaves of both species have been well-characterized (Freitag and Stichler, 2000; Voznesenskaya et al., 2001; Freitag and Stichler, 2002; Voznesenskaya et al., 2002; Akhani et al., 2005), the flowers have not. The types of photosynthesis operating in the stems and flowers of these species were studied by means of anatomical, immunocytochemical, and physiological characterization, and an overview of the floral anatomy was studied by means of light and electron microscopy.

CHAPTER TWO

NON-LEAF CHLORENCHYMA IN *BIENERTIA CYCLOPTERA* AND *SUAEDA ARALOCASPICA* (CHENOPODIACEAE) EXHIBIT SINGLE CELL C_4 PHOTOSYNTHESIS

Introduction

When identifying C_4 plants, Kranz anatomy with its typical spatial separation of photosynthetic enzymes via two distinctive chlorenchyma cell types is expected (Sage, 1999). Recent findings have challenged this paradigm which has existed for 35 years, with the discovery of several plants in the family Chenopodiaceae that undergo traditional C_4 photosynthesis but have a unique anatomy that does not consist of the Kranz dual-cell system (Frietag and Stichler, 2000; 2002). Instead, the single cell C_4 system utilizes individual chlorenchyma cells by means of intracellular biochemical and organelle compartmentation (Voznesenskaya et al., 2001; Voznesenskaya et al., 2002; Edwards et al., 2004).

Among the dicots, the family Chenopodiaceae has the most C_4 species, and large diversity in the types of C_4. There are five types of Kranz anatomy in the family—Atriplicoid, Conspermoid, Kochioid, Salsoloid, and Suaedoid—and two types of C_4 cycles characterized by different C_4 acid decarboxylases, including NAD-malic enzyme (NAD-ME) and NADP-malic enzyme (NADP-ME) (Voznesenskaya and Gamaley, 1986). NAD-ME is localized in mitochondria, while NADP-ME is located in the chloroplasts in bundle sheath cells.

The recently discovered single cell C_4 species in this family occur in subfamily Suaedoideae, and in the genera *Bienertia* and *Suaedae* (Kaprolov et al., 2006). It has been demonstrated that the mature leaves of *Bienertia cycloptera* and *Suaeda aralocaspica*, both members of the family Chenopodiaceae, perform this exceptional form of photosynthesis

(Voznesenskaya et al., 2001; 2002). More recently another species of *Bienertia, B. sinuspersici,* has been discovered which has the same type of C_4 photosynthesis as *B. cycloptera* (Akhani et al., 2005). Leaves make up the majority of the green tissue during vegetative growth of these species, but the young stems are also green, and green flowers are abundant during latter stages of growth. The purpose of this project was to determine by anatomical, immunocytochemical, and physiological characterization whether chlorenchyma tissue in flowers and stems of two single cell C_4 species, *B. cycloptera* and *S. aralocaspica*, have C_3 type chlorenchyma or the unusual structure and mechanism of single cell C_4 photosynthesis.

Materials and Methods

Flowers of various stages ranging from bud to maturity and young, green stems were collected from *Bienertia cycloptera* Bunge ex Boiss and *Suaeda aralocaspica* (Bunge) Freitag & Schutze (Schütze et al., 2003)(=*Borszczowia aralocaspica* Bunge). Plants were grown in a growth chamber with day/night temperatures of 25/18˚C, and a 14/10 h photoperiod, with a stepwise increase and decrease in light intensity during the day to a maximum photosynthetic quantum flux density of 1100 µmol m^{-2} s^{-1}.

For scanning electron microscopy, samples were fixed with 50% (v/v) ethanol, 5% (v/v) acetic acid, and 4% (v/v) formaldehyde, pH 2.9 overnight at 4˚C. They were then post-fixed with 1% (w/v) Osmium tetroxide overnight, dehydrated with a graded ethanol series, dried in a critical point drier (CPD), mounted on stubs, and gold-coated. For ethanol cryofracture, the dehydration steps were followed by the samples being placed in liquid nitrogen, fractured, and then dried in the CPD. All SEM samples were viewed on a Hitachi S-570 scanning electron microscope.

For light microscopy, samples were fixed with 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 50 mM Pipes buffer, pH 7.2 overnight at 4˚C. Following dehydration by a standard procedure the samples were embedded in London Resin White (LR White) acrylic resin. One micrometer thick sections were made on a Reichert Ultracut R ultramicrotome (Austria), and dried onto gelatin-coated slides. For general light microscope observations, samples were stained with Stevenel's Blue. Polysaccharide staining was accomplished by incubating sections in periodic acid (1% w/v) for 30 min, washing, and then incubating in Schiff's reagent (Sigma) for 1 hour.

For in situ immunolocalization, antibodies used were anti-spinach Rubisco (LSU) IgG (courtesy of B. McFadden, Washington State University) and anti-maize PEPC IgG (Chemicon, Temecula, CA, USA). The slides were blocked for 1 hour with 1% bovine serum albumin (BSA) in TBST (10 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.3% (v/v) Tween 20), and then incubated with a 1:500 dilution of the antibody for 3 hours. The slides were washed with TBST and BSA and treated with Protein A-gold (20nm particles diluted 1:50 with TBST and BSA) for 1 hr. The slides were then washed, dried, and exposed to silver enhancement reagent for 15 min following the manufacturer's directions (Amersham). The sections were counterstained with 0.5% (w/v) Safranin O and imaged in a reflected/transmitted mode using a Bio-Rad MRC 1024 confocal system (Biorad, Hercules, CA, USA) mounted on a Nikon Eclipse TE 300 inverted microscope and Lasersharp image program 3.10.

To analyze the potential for flowers to fix atmospheric carbon dioxide, flowers were collected and exposed to $^{14}CO_2$ in the light. For each given time of exposure to $^{14}CO_2$, 10 flowers were collected from plants incubated in a 20 ml vial and flushed with air for 10 min while illuminated with a photosynthetic photon flux density of 1300-1600 µmol quanta m^{-2} s^{-1} at

approximately 25°C. The chamber was then saturated with $^{14}CO_2$ (1.8%) and boiling 95% ethanol was added to stop the reaction following each treatment (1, 3, or 8 min). The flowers were ground in a mortar, the ethanol evaporated, and the pellet resuspended in H_2O and 1 N HCl, and then added to a scintillation vial followed by addition of Biodegradable Counting Scintillant (BCS). The ^{14}C content was measured using a Tri-Carb 1900 TR Liquid Scintillation Analyzer. Ten flowers were exposed to $^{14}CO_2$ in the dark for 8 min as a negative control and the low level of labeling was subtracted from all measurements taken in the light. The chlorophyll content was determined by grinding flowers in 80% acetone and measuring absorbance on a Perkin-Elmer 552A UV/VIS Spectrophotometer. The total amount of chlorophyll a and b was calculated using the following equation:

Chls a + b = (17.76 x $A_{646.6\ nm}$) + (7.34 x $A_{663.6\ nm}$) = µg Chl per ml solution in the spectrophotometer.

For Western blot analysis, proteins were extracted from leaves in 200 mM Bicine-KOH (pH 9.5), 25 mM DTT, and 1% (w/v) SDS. Extracts were boiled for 90 sec with equal volumes of solubilization buffer (62.5 mM Tris, 20% (v/v) glycerol, 2.5% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol, pH 6.8). 20 µg of protein was loaded per lane. Proteins were resolved by SDS-PAGE (Laemmli, 1972) using a linear 7.5-15% acrylamide resolving gel and 5% acrylamide stacking gel. Each gel carried pre-stained SDS-PAGE low molecular weight markers (Bio-Rad). After electrophoresis, proteins on the gel were electro-transferred to nitrocellulose membrane (Towbin et al., 1979) and probed with an appropriate antibody overnight. For antibodies used, see the section on in situ immunolocalization. Goat antirabbit IgG-alkaline phosphatase conjugate (Bio-Rad) was used as the secondary antibody for detection of the enzymes. All blots were air dried and used for image analysis.

Results

The overall floral anatomy of *B. cycloptera* and *S. aralocaspica* is very similar (Fig. 1). The flowers of *B. cycloptera* (Fig. 1A, 2A-C) and *S. aralocaspica* (Fig. 1B, 2D-F) have an outer whorl of five green tepals, and an inner whorl of five, four-lobed anthers, although some monoecious flowers were found (not shown). Each carpel contains a single seed. The stigma and anthers mature simultaneously. Following fertilization, the tepals fuse and the carpel swells to form a fleshy fruit that then dehisces, allowing seed dispersal. Salt glands are present on the abaxial tepal surface of *B. cycloptera* (Fig. 2A, arrows).

Fig. 3A-C shows light microscopy of the tepals of *B. cycloptera.* Fig. 3A shows a cross section of a tepal with a layer of chlorenchyma cells underneath the epidermal tissue on the abaxial side of the leaf which is exposed to the atmosphere in mature flowers. The chlorenchyma cells have a central cytoplasmic compartment with a few chloroplasts around the periphery (Fig. 3A, B), characteristic of the unique chlorenchyma cells previously reported in leaves of this species. Starch is stored in chloroplasts in the central cytoplasmic compartment (Fig. 3C). The epidermal tissue on the adaxial side of the leaf has smaller cells, without any adjacent chlorenchyma tissue.

Fig. 3D-F show light microscopy of tepals of *S. aralocaspica.* The tepals have 1-2 layers of chlorenchyma cells which are positioned towards the abaxial epidermis with a distinct layer of hypodermal cells between the two (Fig. 3D). Young *S. aralocaspica* tepals have rounded hypodermal cells between the epidermis and the chlorenchyma with few air spaces (Fig. 3E), while older tepals have larger air spaces and an elongated hypodermis (Fig. 3D). The chlorenchyma cells have chloroplasts polarized toward opposite ends of the cells (Fig. 3E) which

is characteristic of the leaf chlorenchyma cells in this species (Voznesenskaya et al., 2001). The starch is located towards the proximal end of the cell near the vascular tissue (Fig. 3F).

Sunken stomata are present on the tepals, leaves, and stems of both species (Fig. 4). The surface of *B. cycloptera* flowers (Fig. 4A) and stems (Fig. 4C) is covered by a thick wax which is absent on the *S. aralocaspica* floral epidermis (Fig. 4B). Salt glands are present on *B. cycloptera* tepals (Fig. 4A) and stems (Fig. 4C), and trichomes are also found on *S. aralocaspica* stems (Fig. 4D).

Antibodies to Rubisco and PEPC were used to determine the localization of these two carboxylases in the chlorenchyma cells of young and mature tepals of *B. cycloptera* and *S. aralocaspica*. In *B. cycloptera* there was little or no labeling using preimmune serum (Fig. 5A, D). With antibody to Rubisco there was labeling of chloroplasts throughout the cytoplasm in young tepals while in the mature tepals most labeling was in chloroplasts of the central compartment (Fig. 5B, E). With antibody to PEPC there was labeling throughout the cytoplasm in both young and mature tepals, consistent with its localization in the cytosol.

In *S. aralocaspica* there was no background labeling in the control treatments without Rubisco and PEPC antibody (Fig. 6A, D). In young tepals, labeling with antibody shows Rubisco is located in all chloroplasts which are distributed around the cytoplasm (Fig. 6B), while in chlorenchyma of mature tepals it is preferentially localized in chloroplasts which are polarized toward the proximal end of the cell (towards the vascular tissue) (Fig. 6E). In both young and mature tepals labeling for PEPC is localized in the cytoplasm of chlorenchyma cells (Fig. C, F).

Rates of photosynthesis were determined by measuring light-dependent uptake of $^{14}CO_2$ by mature, green, detached flowers. An increase in ^{14}C content over time was seen in both

species, with rates of 1.19 and 1.14 µmol CO_2 mg Chl^{-1} min^{-1} for *B. cycloptera* and *S. aralocaspica* flowers, respectively (Fig. 7).

Light microscopy was performed on stem sections of *B. cycloptera* and *S. aralocaspica* to identify chlorenchyma tissue. Fig. 8A shows a lower and Fig. 8B a higher magnification of a stem section of *B. cycloptera* in which it is clear that there is a layer of chlorenchyma cells beneath the epidermal tissue. Fig. 8C is a longitudinal section near the surface of the stem which shows many chlorenchyma cells, while Fig. 8D shows a higher magnification. These chlorenchyma are structurally similar to those in the tepals, indicative of single cell C_4 (SC-C_4) photosynthesis, but the cells are discontinuous around the stem. In the stem, Rubisco and PEPC are found in the SC-C_4 chlorenchyma in a similar arrangement to that seen in tepals (Fig. 9). Starch is also present in plastids in cells next to the vascular tissue, but these cells do not have the central cytoplasmic compartment characteristics of SC-C_4 chlorenchyma in these species (Fig. 8E).

In stem sections of *S. aralocaspica*, there is no distinct layer of chlorenchyma cells beneath the epidermal tissue (Fig. 10A, B). Also, there are no chlorenchyma cells which have the distinct polarization of chloroplasts to opposite ends of the cells as occurs in the SC-C_4 type chlorenchyma in leaves and tepals of this species. Rather, there are some chlorenchyma cells scattered throughout the cortical tissue which have chloroplasts around the periphery of the cell typical of C_3 type chlorenchyma (Fig. 10C, D). Starch is present in chlorenchyma cells in the cortical parenchyma (Fig. 10E, F).

Western blots were run on extracts of flowers, leaves, and stems for four photosynthetic enzymes involved in C_4 photosynthesis (PEPC, PPDK, NAD-ME and Rubisco) in these single cell C_4 species, *B. cycloptera* (Fig. 11A) and *S. aralocaspica* (Fig. 11B). In both species, flowers

show similar patterns to leaves with all four enzymes having prominent bands indicative of C_4 photosynthesis. In stems of *B. cycloptera* the intensity of labeling of all four enzymes was much lower than in flowers and leaves, and PPDK was not detected. In *S. aralocaspica*, C_4 enzymes were found in flowers and leaves while stems contained only Rubisco.

Discussion

Kranz anatomy is no longer the only accepted tissue configuration for a C_4 plant. The arrangement of mesophyll and bundle sheath cells that so long has defined C_4 plants is now being joined by efficient single cell C_4 systems as acceptable anatomical alternatives. In two genera of family Chenopodiaceae, *Bienertia* and *Suaeda*, unique forms of C_4 photosynthesis have been discovered. In this study we examined the type of chlorenchyma in flowers and stems of the single cell C_4 species *Bienertia cycloptera* and *Suaeda aralocaspica*. Previously it was shown that both leaves and cotyledons of these species have unique chlorenchyma cells which are structurally and biochemically developed to perform C_4 photosynthesis (Freitag and Stichler, 2000; Voznesenskaya et al., 2001; Freitag and Stichler, 2002; Voznesenskaya et al., 2002; Akhani et al., 2005).

Both *B. cycloptera* and *S. aralocaspica* have green leaves, stems, and flowers. Despite their tiny dimensions, the flowers are quite complex and detailed, anatomically as well as photosynthetically. Five green tepals and five anthers surround the ovary of the flowers. The anthers and stigma mature concurrently allowing self-fertilization to result in a single seed per flower which is protected by the fleshy fruit formed by the tepals and ovary until desiccation.

Though the leaves of both *B. cycloptera* and *S. aralocaspica* are capable of SC-C_4 photosynthesis, the two species have very different means of partitioning the dimorphic

chloroplasts to separate cytoplasmic compartments (Voznesenskaya et al., 2001; 2002). Leaves and cotyledons of *B. cycloptera* have 2 to 3 layers of chlorenchyma cells which surround the water storage and vascular tissue. The chlorenchyma contain chloroplasts in a central, tightly-packed sphere with other chloroplasts scattered around the periphery. Leaves and cotyledons of *S. aralocaspica* have chloroplasts mainly congregated at the proximal end, with a few scattered along the periphery of the cell.

Microscopic analysis of *B. cycloptera* flowers shows photosynthetic cells that are anatomically similar to the leaves (Fig. 3A-C). However, in flowers the chlorenchyma are not continuous but are only on the abaxial side of the tepal, beneath the epidermis. The chlorenchyma cells, which occur in 1-2 layers, are less elongate than those found in leaves. The diameter of the central cytoplasmic compartment is about 15 μm in both organs and thus takes up more of the cell volume in the tepal chlorenchyma. The adaxial surface also has much smaller epidermal cells than the abaxial surface while in leaves the epidermal cell size is constant (Voznesenskaya et al., 2001, 2002). Note here the location of chlorenchyma toward the abaxial side of the leaf which is exposed to the atmosphere where CO_2 can be taken up for photosynthesis through stomata which have been demonstrated (Fig. 4).

In *S. aralocaspica* flowers the chlorenchyma cells have a distinct proximal and distal region as is seen in leaves (Fig. 3D-F). However, the single layer of chlorenchyma cells surrounds only the abaxial side of the vascular tissue while in the leaves chlorenchyma cells are found below the hypodermis around the entire leaf.

The green tissue of the flowers is not only anatomically SC-C_4, bt the chlorenchyma contain compartmentalized photosynthetic enzymes. In *B. cycloptera* leaves, Rubisco is found only in the central chloroplast sphere. PEP carboxylase is found in throughout the cytosol

(Voznesenkaya et al., 2002). The same trend is seen in *B. cycloptera* flowers. Likewise, in *S. aralocaspica* leaves Rubisco is found only in the proximal group of chloroplasts, whereas PEPC is found throughout the cytosol (Voznesenskaya et al., 2001). The flowers contain the same type of enzymatic segregation.

Despite there being anatomical and enzymatic support for photosynthesis in flower tissue, we originally hypothesized that the flowers may merely recycle carbon from flower respiration, rather than fixing carbon from the atmosphere. However, several pieces of evidence indicate that the flowers are fixing atmospheric CO_2. Starch is stored in tepal chlorenchyma cells which likely would not occur if chloroplasts were only assimilating respired CO_2. Since the tepals, like leaves, have prominent stomata, the flowers may be as capable of photosynthesis as leaves. Perhaps most conclusive is the fact that $^{14}CO_2$ is fixed by the flowers at a rate of 1.19 μmol^{14}C/μg Chl · min and 1.14 μmol^{14}C/μg Chl · min for *B. cycloptera* and *S. aralocaspica*, respectively. These rates are similar to rates of photosynthesis with leaves of the two species (Voznesenskaya et al. 2001; 2002).

The stems of *B. cycloptera* include chlorenchyma cells beneath the epidermis which have the SC-C_4 structure both anatomically and enzymatically. This, combined with the discovery of stomata on the stems and the presence of starch in stem chlorenchyma, suggests the fixation of atmospheric CO_2 by stems through the SC-C_4 system. Some cells lacking in SC-C_4 anatomy that directly surround the vascular tissue also contain starch, which suggests that starch-storing plastids are present. If photosynthetic, e.g. via C_3 cycle, they may refix respired CO_2 around the vascular tissue. The stems of *S. aralocaspica* have chlorenchyma cells scattered throughout the cortical tissue with chloroplasts around the periphery of the cell, typical of C_3 type chlorenchyma.

No stem cells with the SC-C_4 anatomy were found, leading to the conclusion that stem tissue does not have SC-C_4 chlorenchyma.

These results, combined with previous studies, indicate that *B. cycloptera* assimilates atmospheric CO_2 throughout its life cycle via SC-C_4 photosynthesis. In natural habitats in Central Asia it germinates in the spring with cotyledons as the first photosynthetic tissue, then develops vegetative growth through photosynthesis by leaves and young stems, and finally reproductive growth in the fall with photosynthesis by flowers which could contribute to seed development (Akhani et al., 2003). Likewise, *S. aralocaspica* cotyledons, leaves, and flowers (but not stems) perform SC-C_4 photosynthesis through its life cycle.

In summary, different photosynthetic types, C_4, C_3, CAM, are usually identified according to the type of photosynthesis in leaves. However, it is of interest to determine whether other photosynthetic organs have the same or different types of chlorenchyma cells and photosynthesis. Not all C_4 plants have leaves as the primary photosynthetic organ during vegetative growth. For example, C_4 species of *Haloxylon* and *Halosarcia* in family Chenopodiaceae have primitive leaves with stems as the primary photosynthetic organ (Ocallaghan, 1992; Pyankov et al., 2000a). Among C_4 chenopods with C_4 leaves there is variation between species as to the type of photosynthesis in cotyledons, with some species having cotyledons with C_4 anatomy and biochemistry, and others having cotyledons which are C_3 in anatomy and function (Butnik, 1979; 1984; Butnik et al., 1991; Pyankov et al., 2000a; 2000b; Voznesenskaya et al., 2004).

Studies on the occurrence and mechanism of photosynthesis in other organs, e.g. flowers and stems, among C_4 chenopods are generally lacking. The present study on two different types of SC-C_4 systems (*B. cycloptera* versus *S. aralocaspica*) shows that the flower tepals have the same type of SC-C_4 chlorenchyma cells as those in leaves and cotyledons. While *B. cycloptera*

also has C_4 type chlorenchyma in the stem, *S. aralocaspica* has C_3 type chlorenchyma tissue in the stem. Where different types of photosynthesis occur in different organs of a given species it is of interest to consider how this is genetically controlled, and what advantage it may be relative to environmental conditions in its habitat during its life cycle. Further studies are needed on the C_4 chenopods to determine the diversity in occurrence and function of photosynthesis in different organs.

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Fig. 1. A single, mature flower of (A) *Bienertia cycloptera* and (B) *Suaeda aralocaspica*. Scale bars, A = 1 mm, B = 1 mm.

Figure abbreviations: a, anther; st, stigma; t, tepal.

Fig. 2. (A-C) Scanning electron micrographs (SEM) of *Bienertia cycloptera* flowers. (A) Tepals on bud beginning to open as the anthers and stigma mature (B) and closing once the stigma is fully extended (C). Salt glands (sg) are present on the abaxial surface of the tepals (A). (D-F) SEM pictures of *Suaeda aralocaspica* flowers. Bud tepals are closed (D), but then open as the anthers and stigma mature (E) and close once the stigma is fully extended (F). Scale bars, A = 0.5 mm, B = 0.5 mm, C = 0.5 mm, D = 0.3 mm, E = 0.6 mm, F = 0.4 mm.

Figure abbreviations: a, anther; s, stigma; sg, salt gland; t, tepal; tr, trichome.

Fig. 3. General anatomy of *Bienertia cycloptera* (A-C) and *Suaeda aralocaspica* (D-F). (A) *Bienertia cycloptera* tepal cross-section with chlorenchyma cells (ch) directly beneath epidermal tissue (e). (B) Longitudinal section of *Bienertia cycloptera* tepal showing nucleus (n) and central cytoplasmic compartment (ccc). (C) Periodic acid-Schiff's (PAS) staining for carbohydrates in *Bienertia cycloptera* tepal cross-section. (D) *Suaeda aralocaspica* tepal longitudinal section with chlorenchyma cells (ch) surrounding the abaxial side of the vascular bundles (vb). (E) Longitudinal section of *Suaeda aralocaspica* tepal showing one layer of hypodermal (h) cells above a single layer of chlorenchyma (ch). (F) Periodic acid-Schiff's staining for carbohydrates in tepal longitudinal section of *Suaeda aralocaspica*. Scale bars, A = 50 µm, B = 25 µm, C = 50 µm, D = 50 µm, E = 50 µm, F = 50 µm.

Figure abbreviations: ccc, central cytoplasmic compartment; ch, chlorenchyma; e, epidermis; h, hypodermis; n, nucleus; s, starch grains; vb, vascular bundles; ws, water storage tissue.

Fig. 4. SEM pictures of stomates on (A) *Bienertia cycloptera* and (B) *Suaeda aralocaspica* tepals. Scale bars, A = 100 µm, insert = 15 µm, B = 50 µm, insert = 12 µm, C = 100 µm, insert = 15 µm, D = 100 µm, insert = 15 µm.

Figure abbreviations: sg, salt gland; sto, stomates; tr, trichome.

Fig. 5. Reflected/transmitted confocal imaging of in situ immunolocalization of photosynthetic enzymes in tepal cross-sections of *Bienertia cycloptera*. Label appears as red particles. (A-C) young tepal (epidermal tissue displaced during fixation) and (D-F) mature tepal. (A, D) control, (B, E) rubisco, and (C, F) PEPC. Scale bars = 25 µm.

Figure abbreviations: ch, chlorenchyma; e, epidermis; ws, water storage.

Fig. 6. Reflected/transmitted confocal imaging of in situ immunolocalization of photosynthetic enzymes in longitudinal tepal sections of *Suaeda aralocaspica*. Label appears as red particles. (A-C) young tepal and (D-F) mature tepal. (A, D) control, (B, E) rubisco, and (C, F) PEPC. Scale bars = 20 µm.

Figure abbreviations: ch, chlorenchyma; e, epidermis; h, hypodermis; vb, vascular bundle.

Fig. 7. (A) Uptake of $^{14}CO_2$ by mature *Bienertia cycloptera* flowers. (B) Uptake of $^{14}CO_2$ by *Suaeda aralocaspica* flowers with open tepals.

Fig. 8. Stem of *Bienertia cycloptera*. (A) General anatomy of stem cross-section with chlorenchyma cells (ch) at discrete intervals beneath the epidermis. (B) Cross-section showing chlorenchyma (ch) directly beneath the epidermis (e), surrounding cortical parenchyma (cp) and vascular bundles (vb). (C) Longitudinal section showing chlorenchyma (ch) in separate areas of the stem. (D) Longitudinal section showing layers of chlorenchyma cells with central cytoplasmic compartments (ccc) beneath the epidermis. (E) PAS staining showing starch in chlorenchyma cells next to the epidermis (ch) and outside the vascular bundles (vb). (F) PAS staining of starch in chlorenchyma (ch). Scale bars, A = 100 µm, B = 100 µm, C = 50 µm, D = 25 µm, E = 50 µm, F = 50 µm.

Figure abbreviations: ccc, central cytoplasmic compartment; ch, chlorenchyma; cp, cortical parenchyma; e, epidermis; pc, peripheral chloroplasts; s, starch grains; sto, stomata; vb, vascular bundles.

Fig. 9. Reflected/transmitted confocal imaging of in situ immunolocalization of photosynthetic enzymes in stem cross-sections of *Bienertia cycloptera*. Label appears as red particles. (A) control, (B) rubisco, and (C) PEPC. Scale bars = 25 µm.

Figure abbreviations: ch, chlorenchyma; e, epidermis.

Fig. 10. Stem of *Suaeda aralocaspica*. (A) General anatomy of stem cross-section with vascular bundles (vb) surrounded by cortical parenchyma (cp). (B) Longitudinal section showing cortical parenchyma cells between the epidermis (e) and the vascular bundles (vb). (C) Cross-section showing scattered chloroplasts in cortical parenchyma (cp) cells around the vascular bundle (vb). (D) Longitudinal cortical parenchyma cells containing scattered chloroplasts. (E) PAS stain showing starch grains mainly in cortical parenchyma (cp) surrounding vascular bundles (vb)in cross-section. (F) PAS stain of longitudinal section showing starch grains clustered in cortical parenchyma cells. Scale bars, A = 200 µm, B = 50 µm, C = 50 µm, D = 25 µm, E = 25 µm, F = 25 µm.

Figure abbreviations: cp, cortical parenchyma; e, epidermis; s, starch; vb, vascular bundles.

Fig. 11. Immunoblots for PEPC, PPDK, NAD-ME, and Rubisco using 10 µg per lane total soluble protein extracted from flowers, leaves, and stems of (A) *Bienertia cycloptera* and (B) *Suaeda aralocaspica.*

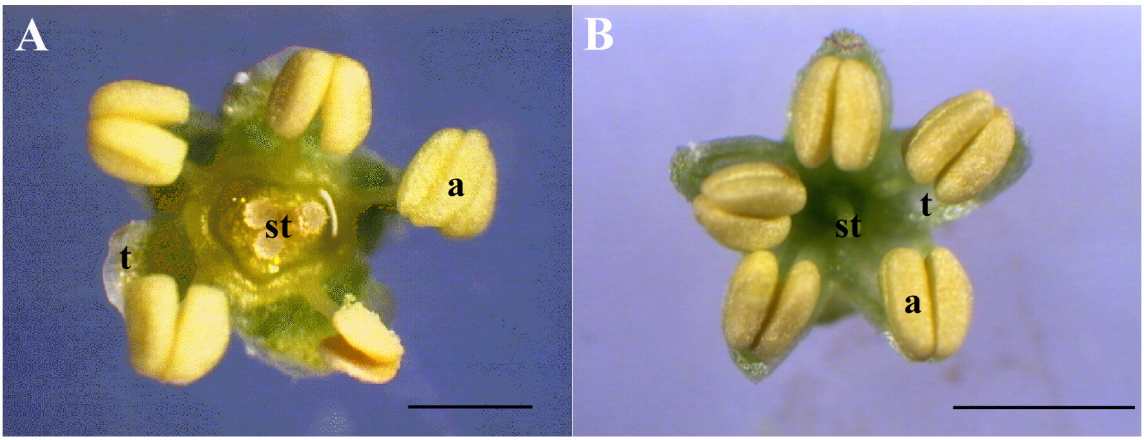

Fig. 1

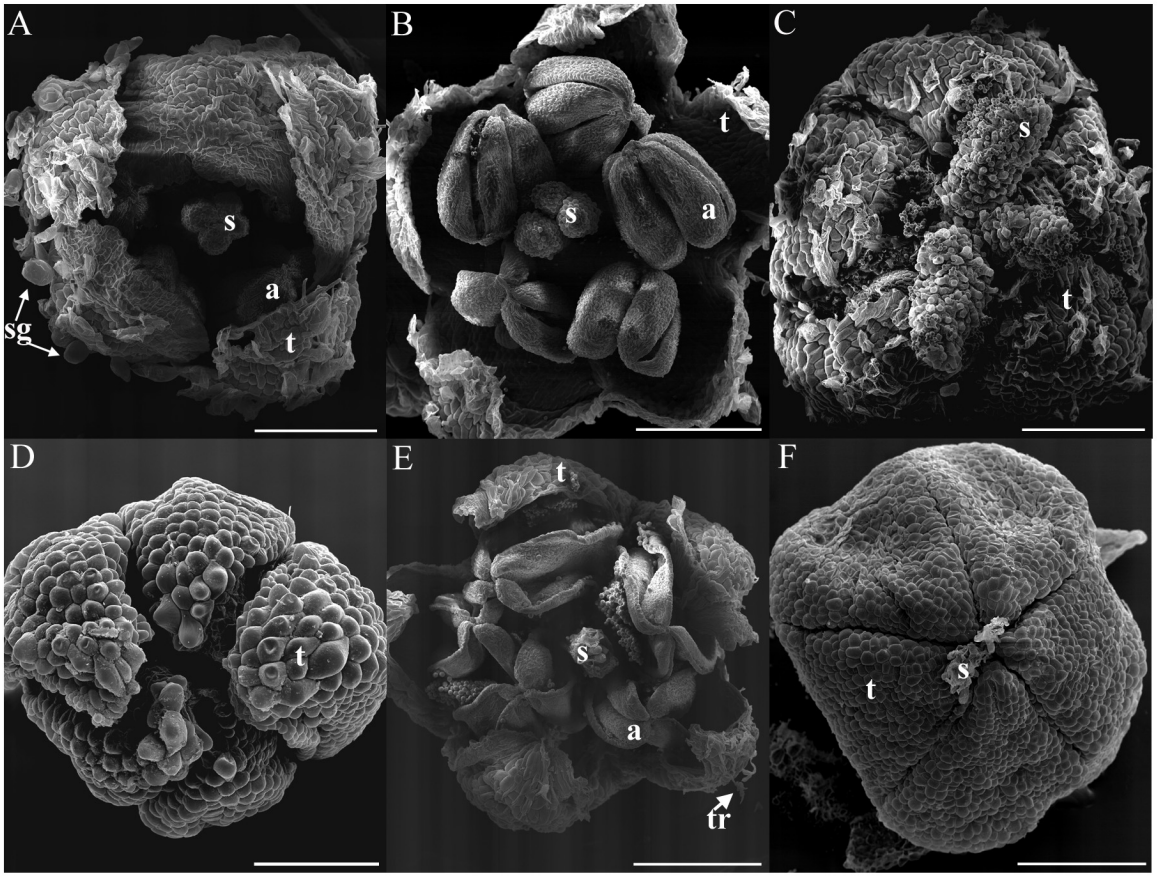

Fig. 2

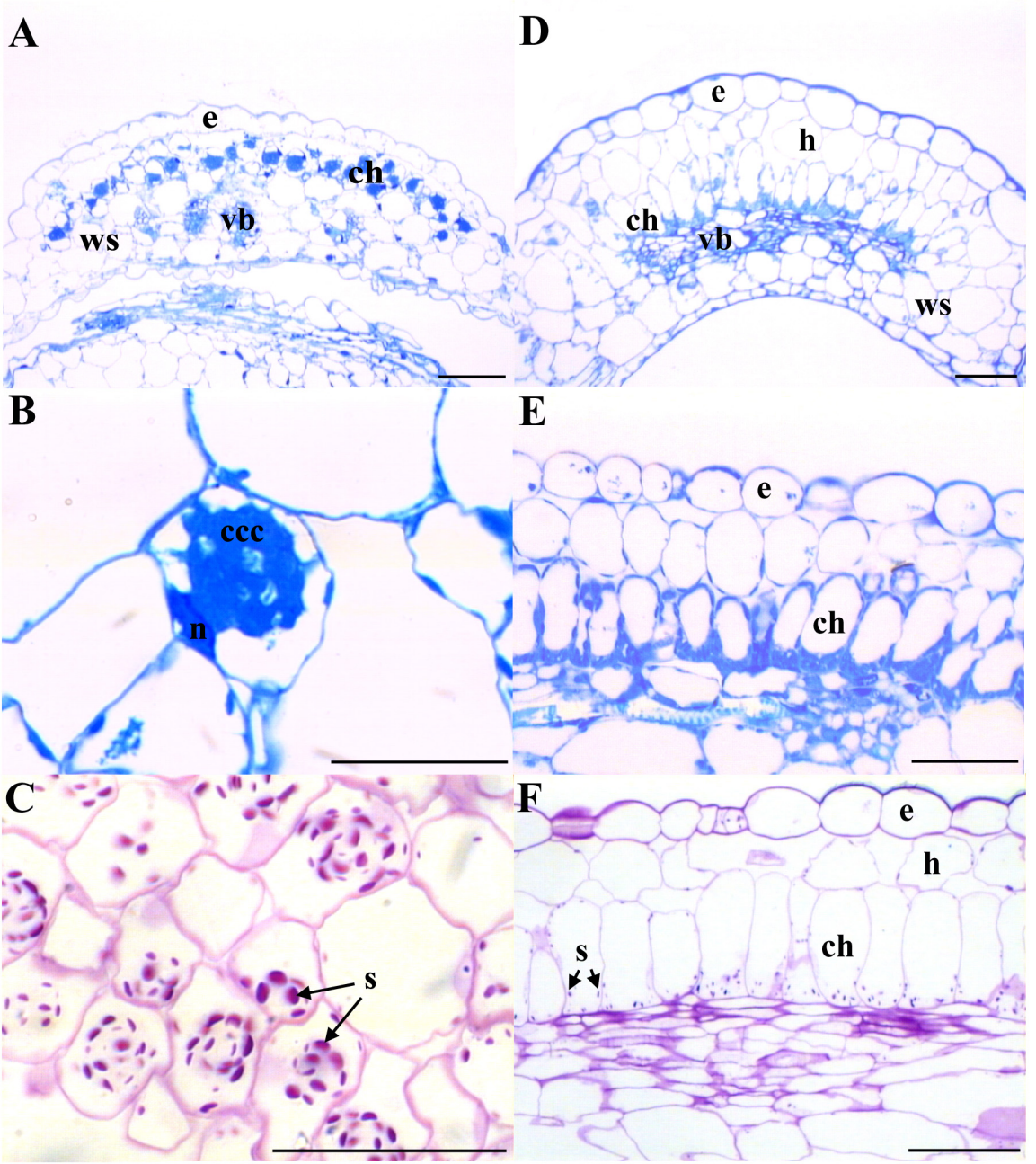

Fig. 3

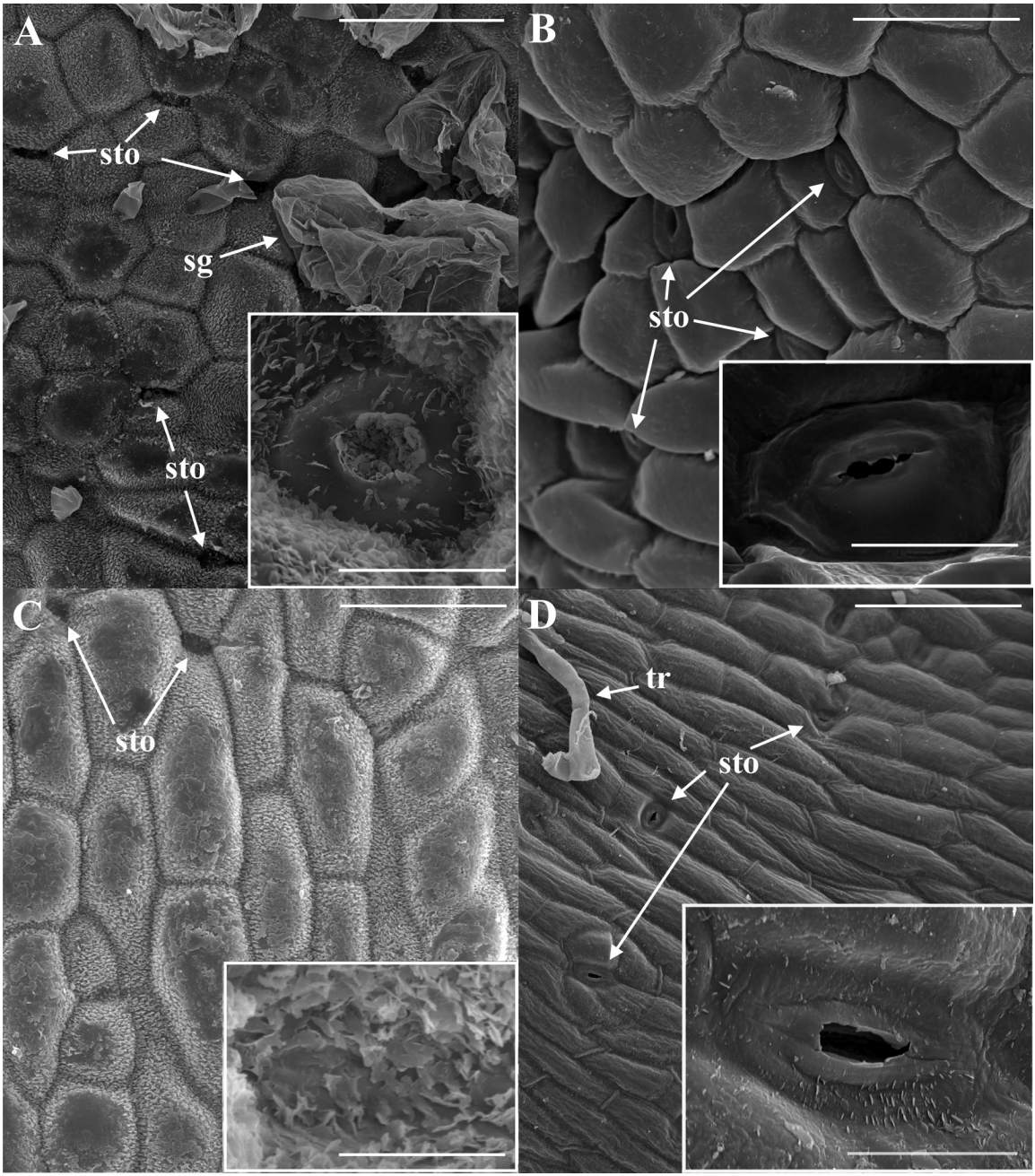

Fig. 4

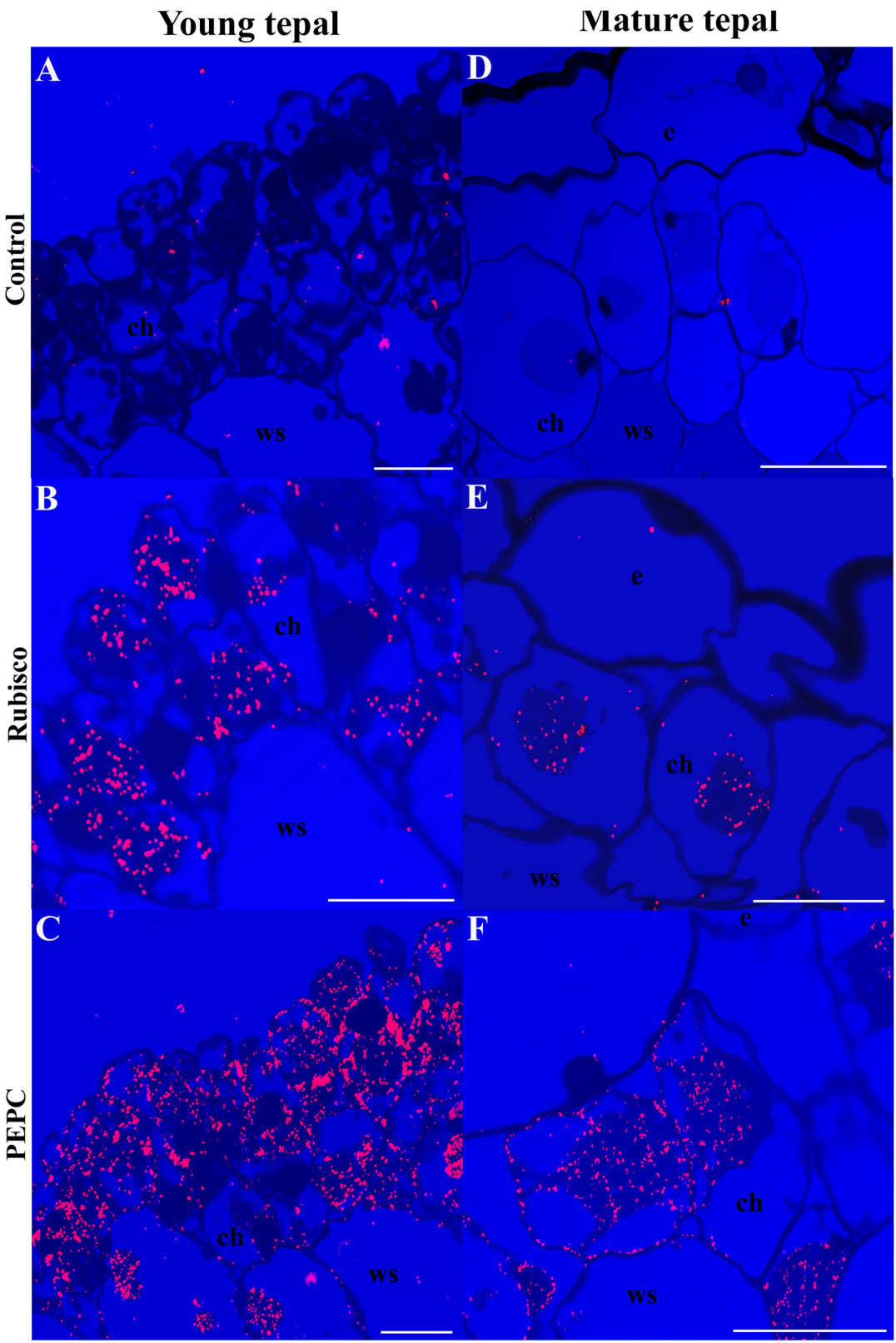
Young tepal
Mature tepal
Control
Rubisco
PEPC
A
B
C
D
E
F
ch
ws
e

Fig. 5

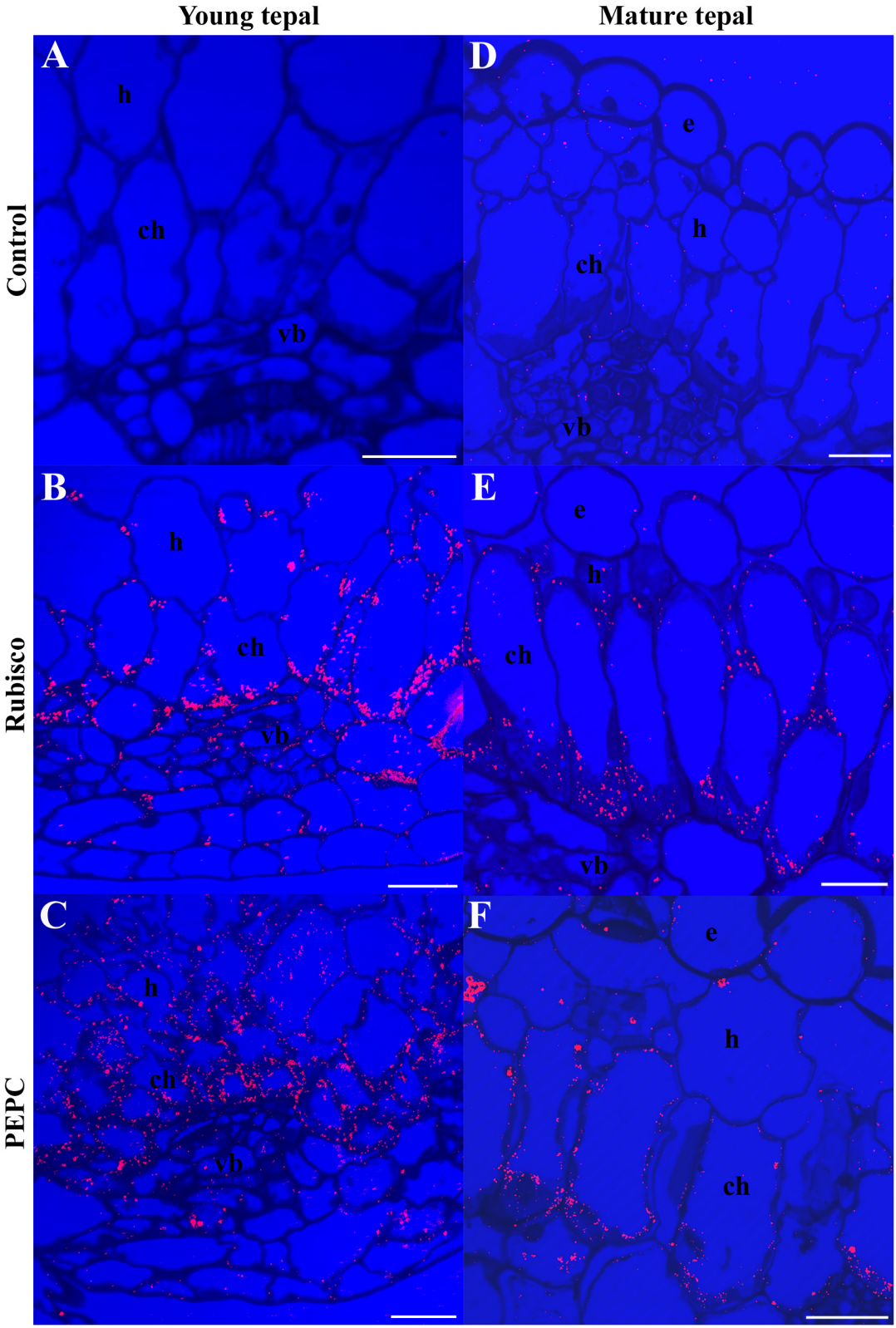
Young tepal
Mature tepal
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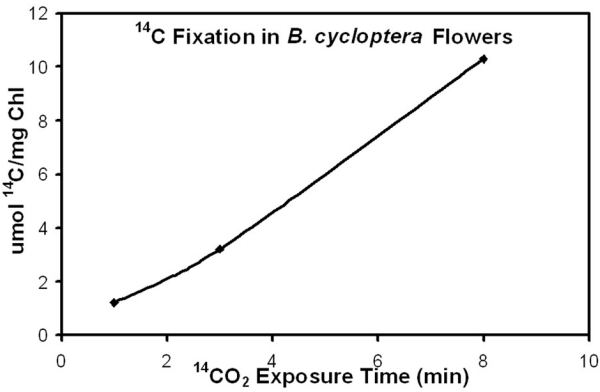
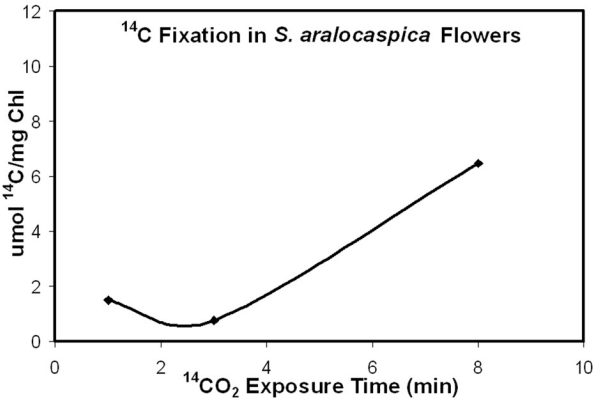
Fig. 6


^{14}C Fixation in *B. cycloptera* Flowers
umol ^{14}C/mg Chl
$^{14}CO_2$ Exposure Time (min)
0
2
4
6
8
10
12


^{14}C Fixation in *S. aralocaspica* Flowers
umol ^{14}C/mg Chl
$^{14}CO_2$ Exposure Time (min)
0
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12

Fig. 7

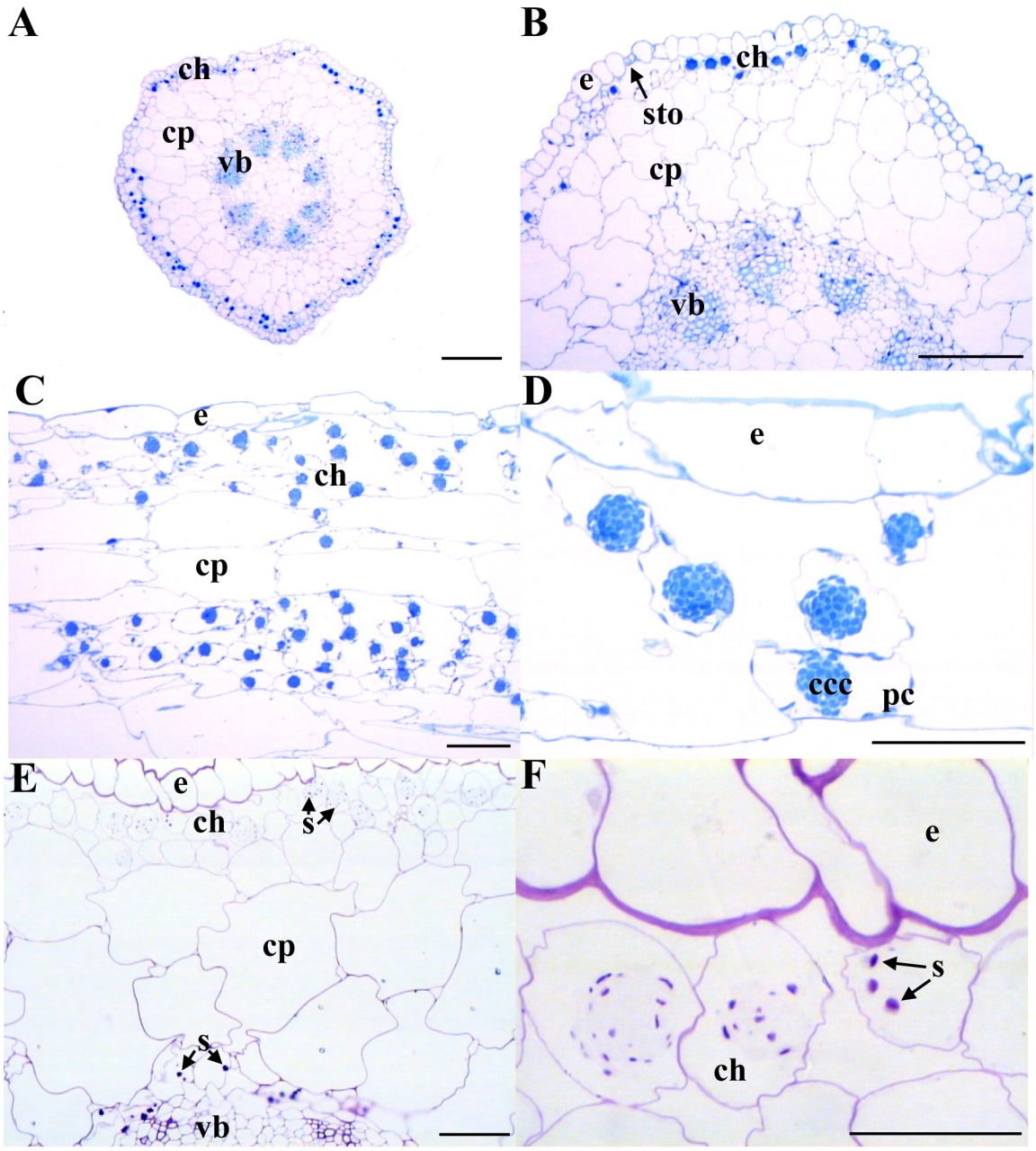


Fig. 8

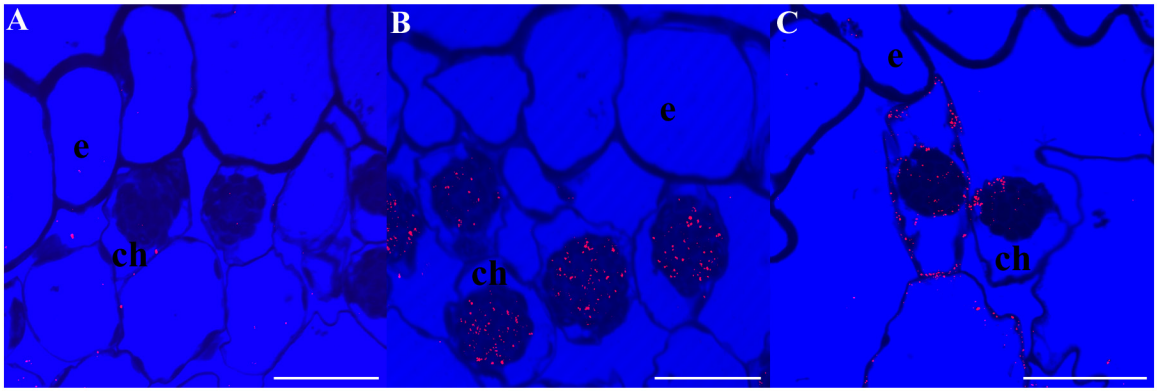


Fig. 9

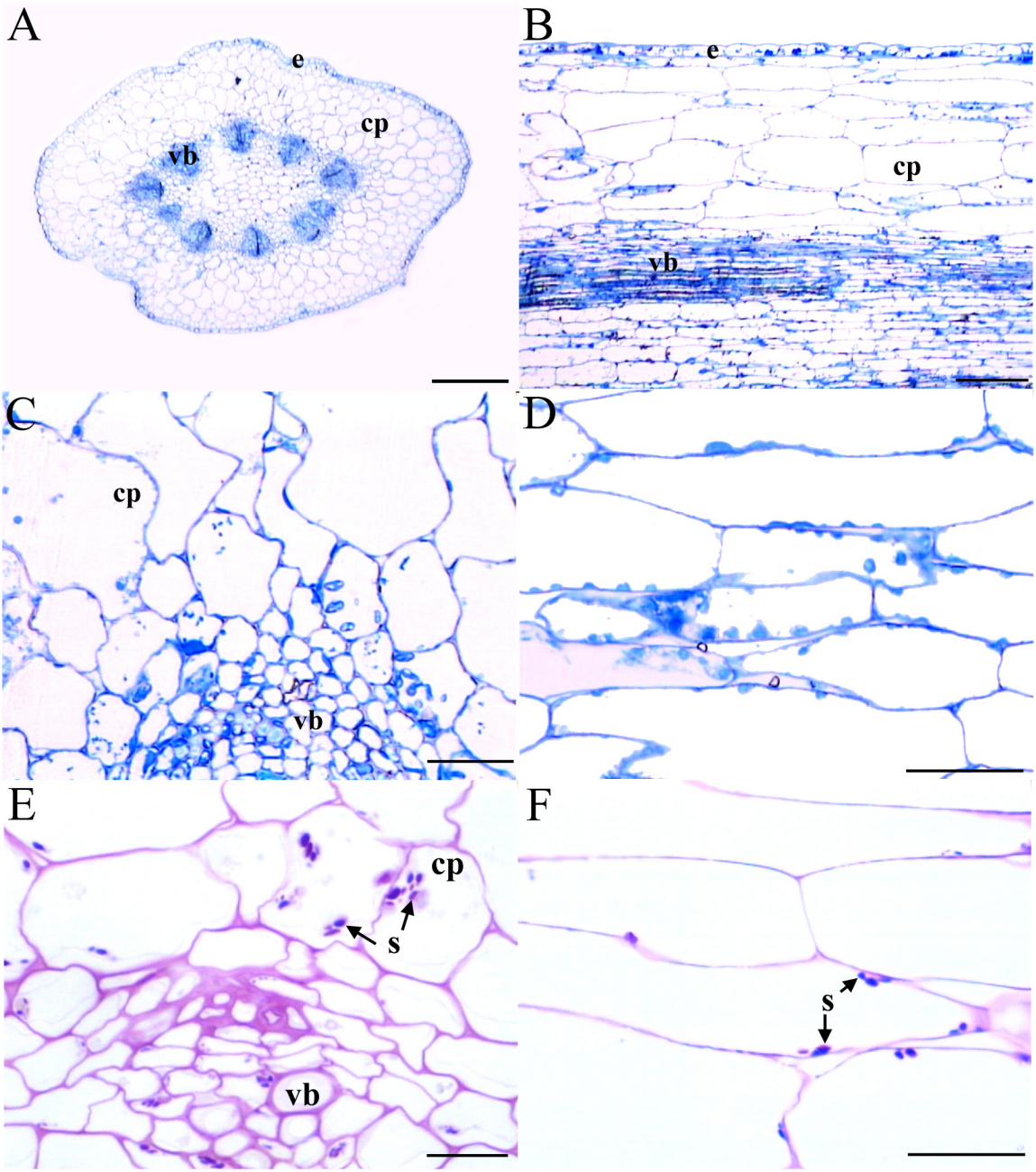


Fig. 10

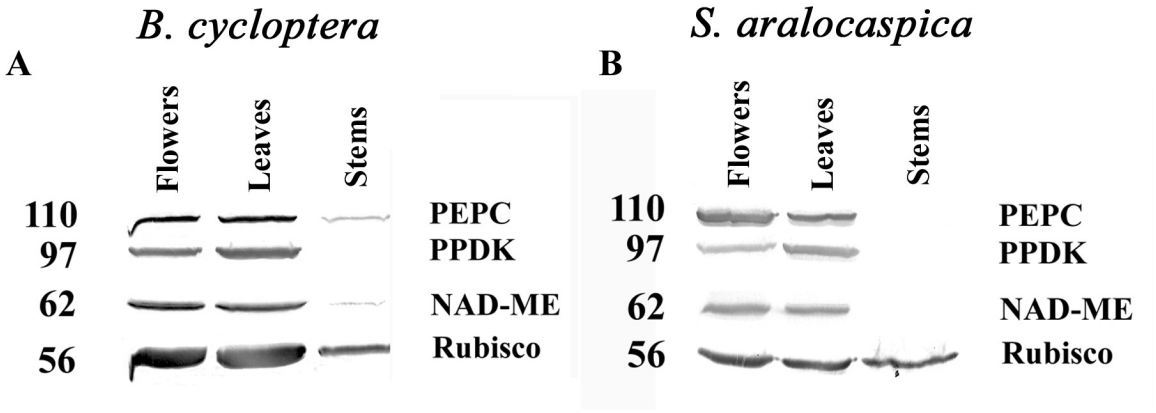
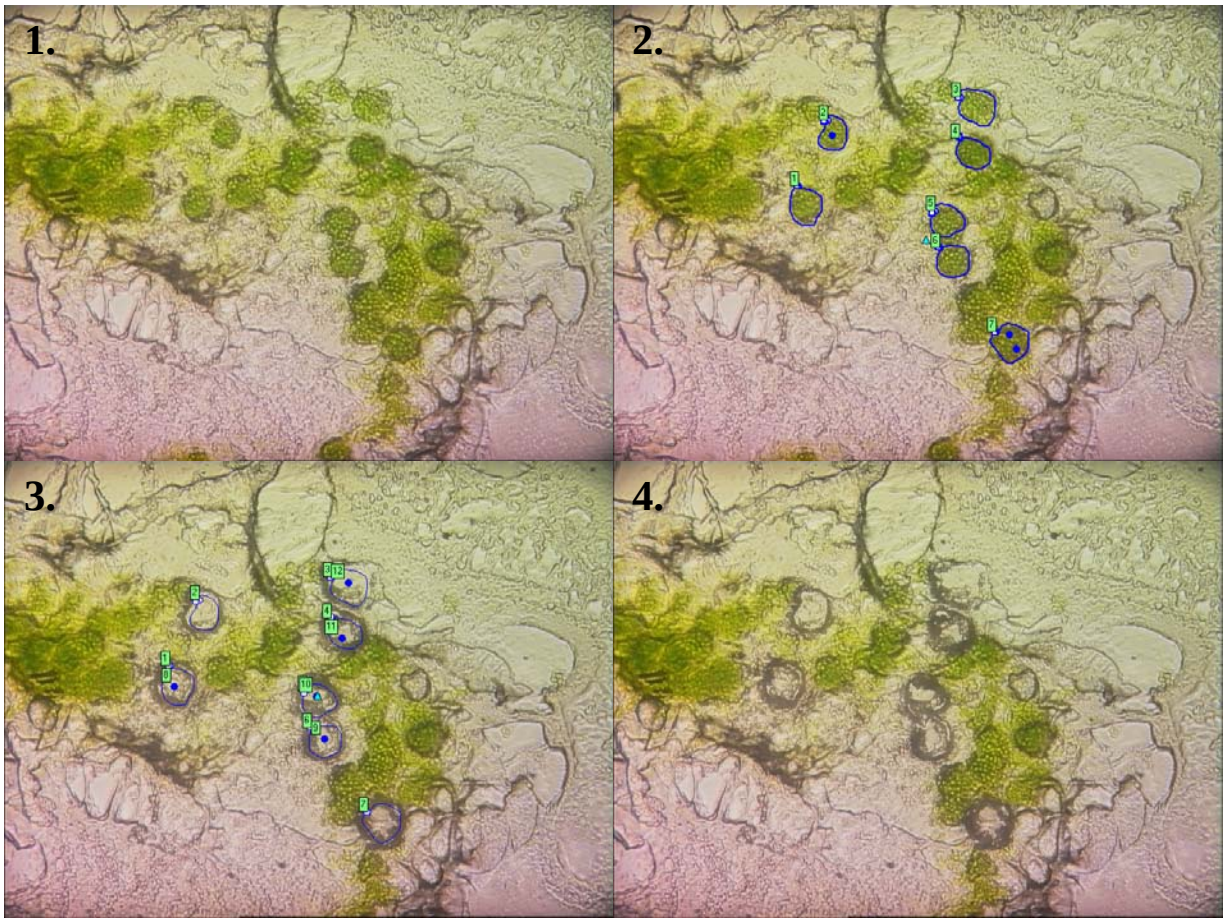

B. cycloptera
A
Flowers
Leaves
Stems
110
97
62
56
PEPC
PPDK
NAD-ME
Rubisco
S. aralocaspica
B
Flowers
Leaves
Stems
110
97
62
56
PEPC
PPDK
NAD-ME
Rubisco

Fig. 11

Laser capture microdissection of single cell C_4 plant chlorenchyma cells

Bienertia sinuspersici is one of the three known single cell C_4 species that conduct C_4 photosynthesis in a single cell via enzyme and organelle compartmentation. For researchers, the single cell system has the potential to allow the engineering of C_4 photosynthesis in C_3 plants without having to engineer C_4 Kranz anatomy. Finding the mechanism that triggers the enzymatic compartmentation would greatly aid this process. Either mRNA trafficking sends transcripts to specific destinations, or the enzymes themselves must be targeted to the compartments. In order to determine whether the enzymes are compartmentalized by mRNA or protein trafficking, laser capture microdissection was used to separate and collect the two compartments of individual cells using conditions that prevented mRNA degradation.

Fresh leaves were sectioned on a Cryocut 1800, Reichert-Jung cryostat at -17˚C and stored at -80˚C until needed. The cell compartments remained intact when 16 µm sections were taken. Cells were microdissected using a PALM Robo and collected in a solution containing RNAse inhibitor. The central cytoplasmic compartments were first collected (see below), generally 500 per treatment which took approximately one hour. Then the remaining peripheral cytoplasmic compartments were collected. Although inconsistency of RT-PCR results prevented further research to address questions of interest, it was clear that this technique can be applied to separating the two cytoplasmic compartments.



Cross-section of *B. sinuspersici* leaf before (1, 2) and after (3, 4) laser capture microdissection